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NY-ESO-1 mRNA expression and immunogenicity in advanced prostate cancer

Tetsuya Nakada[1,2], Yuji Noguchi[1], Shuichiro Satoh[1], Toshiro Ono[1], Takashi Saika[2], Takushi Kurashige[2], Sacha Gnjatic[3], Gerd Ritter[3], Yao-Tseng Chen[3,4], Elisabeth Stockert[3*], Yasutomo Nasu[2], Tomoyasu Tsushima[2], Hiromi Kumon[2], Lloyd J. Old[3], and Eiichi Nakayama[1]

[1]Department of Immunology, Okayama University Graduate School of Medicine and Dentistry, Japan
[2]Department of Urology, Okayama University Graduate School of Medicine and Dentistry, Japan
[3]Ludwig Institute for Cancer Research, New York Branch at Memorial Sloan-Kettering Cancer Center, New York, NY, USA
[4]Department of Pathology, Cornell University Medical College, New York, NY, USA
[*]Deceased September 21, 2002

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Abstract

NY-ESO-1 mRNA expression was investigated in advanced prostate cancer by conventional and quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR). *NY-ESO-1* mRNA was detected in 20 of 53 (38%) tumor specimens. Four of 15 (27%) stage C, 1 of 3 stage D1 (33%) and 15 of 35 (43%) stage D2 prostate cancers were positive. The presence of NY-ESO-1 antibodies was evaluated in sera from a panel of 218 patients with prostate cancer, including the 53 patients whose tumors were examined for *NY-ESO-1* mRNA expression. NY-ESO-1 antibodies were detected in 1 of 30 (3.3%) stage D1 and 9 of 110 (8.2%) stage D2 patients, whereas none of the 78 patients with localized prostate cancer (stages A, B and C) had detectable NY-ESO-1 antibodies. Of the 53 patients whose tumors were examined for *NY-ESO-1* mRNA expression, 2 of 20 patients with *NY-ESO-1* mRNA-positive tumors had NY-ESO-1 antibodies. No antibody was found in the sera of 32 patients with *NY-ESO-1* mRNA-negative tumors, with the exception of one patient with regional lymph node metastasis (stage D1). CD8 T cell responses specific to NY-ESO-1 were detected in two of three patients with NY-ESO-1 antibodies.

Introduction

Prostate cancer ranks first in incidence among malignancies of men in the United States (1) and the number of patients diagnosed with prostate cancer has been increasing in Japan (2). Although androgen ablation therapy is highly effective, metastatic prostate cancer eventually becomes hormone-refractory. Chemotherapy has been shown to have little or no impact on survival of patients with hormone-refractory prostate cancer (3, 4). The

median survival of patients with hormone-refractory prostate cancer is approximately 9 to 12 months and approximately 37,000 patients in the United States and 7,000 in Japan die annually (1, 2).

The number of human tumor antigens recognized by CD4/CD8 T cells or antibodies has been increasing for the last decade (5). Cancer/testis (CT) antigens have attracted attention as potent targets for immunotherapy against cancer because of their restricted expression in normal tissues and their expression in a wide variety of cancers (6). Of the more than ten gene or gene families coding for CT antigens (7), *NY-ESO-1* appears to be the most immunogenic and has been shown to induce humoral immune responses and specific CD8 T cell reactivity in approximately half of the patients with advanced *NY-ESO-1* mRNA-positive malignant melanoma (8, 9). The frequency of *NY-ESO-1* mRNA expression varies depending on the types of cancer; for instance, it is high in synovial sarcoma, intermediate in transitional cell carcinoma, melanoma and lung cancer, and low in colon and renal cancer (10, 11, 12).

In this study, we found high expression of *NY-ESO-1* mRNA in advanced prostate cancer. Moreover, analysis of sera from prostate cancer patients for the presence of NY-ESO-1 antibodies showed that seropositivity was restricted to patients with lymph node or systemic metastasis. In two of three patients with NY-ESO-1 antibodies, CD8 T cell responses specific to NY-ESO-1 were observed.

Results

Expression of *NY-ESO-1* mRNA in advanced prostate cancer

Expression of *NY-ESO-1* mRNA in prostate cancer specimens was investigated by RT-PCR. Tumor specimens used for mRNA preparation were obtained from 53 patients with hard nodular lesions palpable by digital rectal examination. The tumors were clinically classified as stage C (15/53) or D (38/53) prostate cancer. *NY-ESO-1* mRNA was detected in 20/53 (38%) tumor specimens as shown in Table 1. The size of the PCR product in tumors was the same as that in testis, as shown in Figure 1A, and the PCR product was confirmed to be *NY-ESO-1* by nucleotide sequencing. No *NY-ESO-1* mRNA expression was observed in 8 benign prostatic hyperplasia (BPH) specimens (Table 1 and Figure 1). *NY-ESO-1* mRNA expression was also determined by quantitative real-time RT-PCR using the *NY-ESO-1*-specific primers and TaqMan probe. Twenty prostate cancer specimens that were positive for *NY-ESO-1* mRNA expression by conventional RT-PCR showed expression of *NY-ESO-1* mRNA at levels that varied from 3.4% to 550% (Figure 1B) relative to the level in normal testis. No expression of *NY-ESO-1* mRNA was observed in 8 BPH and 33 prostate cancer specimens that were negative for *NY-ESO-1* mRNA expression by conventional RT-PCR.

The relationship between *NY-ESO-1* mRNA expression and pathological and clinical features is shown in Table 1. Prostate cancer with bone metastasis (stage D2) appeared to express *NY-ESO-1* mRNA more frequently than localized prostate cancer (stage C) or prostate cancer with lymph node metastasis (stage D1), although the difference was not statistically significant. No correlation was observed between *NY-ESO-1* mRNA expression and the patients' age, serum prostate specific antigen (PSA) level and Gleason's score.

Table 1. Correlation between *NY-ESO-1* mRNA expression and pathological and clinical features in advanced prostate cancer.

Pathological and Clinical Features		NY-ESO-1 mRNA Positive Tumors	
		Proportion	Percentage
Benign prostatic hyperplasia		0/8	0%
All tumor specimens		20/53	38%
Gleason's score	less than or equal to 7	6/21	29%
	greater than or equal to 8	14/32	44%
Clinical stage	C	4/15	27%
	D1	1/3	33%
	D2	15/35	43%

Antibody responses to NY-ESO-1 in prostate cancer patients

Sera from 218 prostate cancer patients who were diagnosed histologically at the Okayama University Hospital from January 1999 to December 2002, including the 53 patients whose tumors were examined for *NY-ESO-1* mRNA expression, were assayed for the presence of NY-ESO-1 antibodies by ELISA using recombinant NY-ESO-1 protein as the antigen. Figure 2 shows typical titration curves for NY-ESO-1 antibody positive and negative sera, and Table 2 summarizes the results. The frequency of stage A, B, C and D prostate cancer at the time of diagnosis in this panel of 218 patients was 4.1%, 12.9%, 18.8% and 64.2% respectively. NY-ESO-1 antibodies were found only in patients with metastatic disease (stages D1 and D2, 10/140), but not in patients with localized tumors (stages A, B and C, 0/78). Of the patients with stage D prostate cancer, NY-ESO-1 antibodies were found in the sera from 1 of 30 (3.3%) stage D1 and 9 of 110 (8.2%) stage D2 patients. No antibody was detected in the sera from 32 healthy volunteers (data not shown). In the 53 patients whose tumors were examined for *NY-ESO-1* mRNA expression, 2 of 20 patients with *NY-ESO-1* mRNA-positive tumors produced NY-ESO-1 antibodies (Table 3). No NY-ESO-1 antibody was detected in 32 patients with *NY-ESO-1* mRNA-negative tumors. Seropositivity was observed for the serum from one patient with an *NY-ESO-1* mRNA-negative primary tumor; the *NY-ESO-1* mRNA expression status in the regional lymph node metastasis of this patient could not be determined.

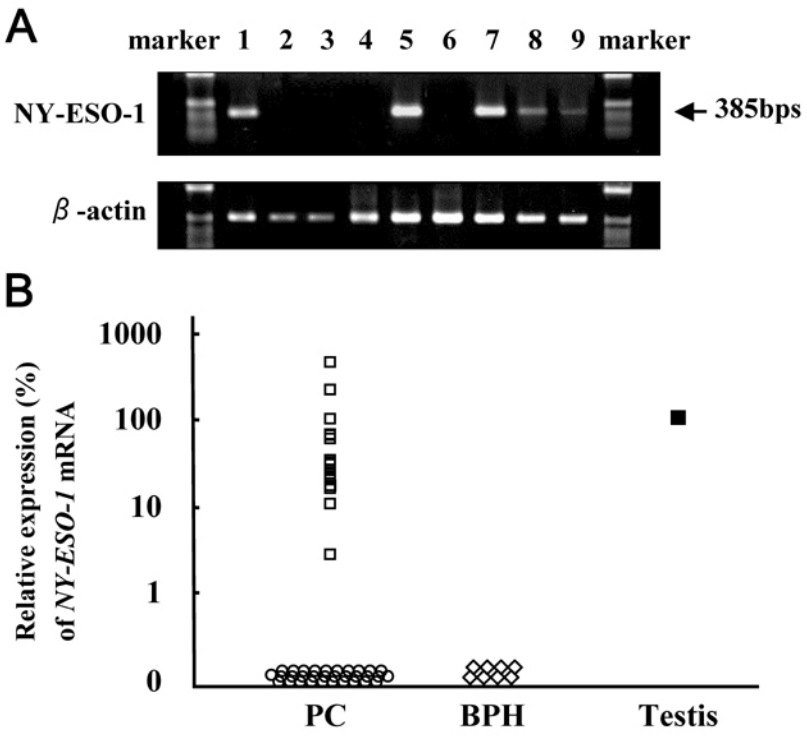

Figure 1. Expression of *NY-ESO-1* mRNA in benign prostatic hyperplasia and prostate cancer. (A) Conventional RT-PCR analysis for *NY-ESO-1* mRNA expression in BPH and prostate cancer: mRNA from testis (lane 1), BPH (lanes 2 and 3), stage C prostate cancer (lanes 4 and 5), stage D1 prostate cancer (lane 6), stage D2 prostate cancer (lanes 7 and 8), a bone metastatic lesion (lane 9) of the tumor in lane 8. (B) Quantitative real-time RT-PCR analysis for *NY-ESO-1* mRNA expression in BPH and prostate cancer. Twenty (open square) and 33 prostate cancer specimens (open circle) which were positive and negative for *NY-ESO-1* mRNA expression respectively by conventional RT-PCR and 8 BPH specimens (open diamond) were analyzed by real-time RT-PCR. The expression relative to testis (filled square) was determined as described.

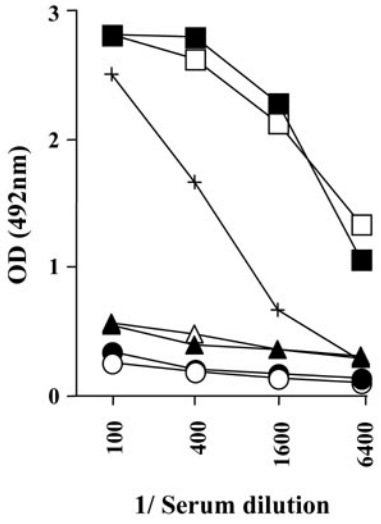

Figure 2. NY-ESO-1 antibodies in patients with prostate cancer. ELISA tests were performed with recombinant NY-ESO-1 protein as the antigen: Serially diluted sera from patients with *NY-ESO-1* mRNA positive (filled square, open square) or negative (filled triangle, open triangle) tumors, positive control serum from an NY-ESO-1 positive malignant melanoma patient (cross), and negative control sera from healthy volunteers (filled circle, open circle).

Table 2. NY-ESO-1 antibody responses in 218 patients with prostate cancer[a].

Clinical Stage	Antibody Positive Sera	
	Proportion	Percentage
A	0/9	0%
B	0/28	0%
C	0/41	0%
D1	1/30	3.3%
D2	9/110	8.2%
Total	**10/218**	**4.6%**

[a]The difference in the frequency of detection of NY-ESO-1 antibody between patients with metastasis (stages D1 and D2, 10/140) and without metastasis (stages A, B, and C, 0/78) was statistically significant ($P<0.05$). Statistical analysis was performed using Fisher's exact test.

CD8 T cell responses specific for NY-ESO-1 in prostate cancer patients

CD8 T cell responses to NY-ESO-1 were evaluated using recombinant viral constructs encoding *NY-ESO-1* cDNA in 3 seropositive and 1 seronegative patients with stage D prostate cancer (Table 4). Purified CD8 T cells from PBLs were stimulated with autologous CD8-depleted PBLs infected with Ad2/ESO to recall memory CD8 T cell responses to NY-ESO-1 (13). Figure 3 shows the results of IFN-gamma ELISPOT assays using autologous B cells infected with recombinant vaccinia viruses as targets. The number of IFN-gamma ELISPOTs produced by $1x10^5$ CD8 T cells are shown in Figure 3A. In two seropositive patients designated PC-1 and -2, CD8 T cells efficiently produced IFN-gamma ELISPOTs in response to autologous B cells infected with vaccinia/*NY-ESO-1* recombinant virus (v.v. ESO), but not wild-type vaccinia virus (v.v. WT). No specific CD8 T cell response to NY-ESO-1 was observed in seropositive patient PC-3 and no definitive response was seen in seronegative patient PC-4. IFN-gamma ELISPOTs produced by effector CD8 T cells from 2 seropositive patients were further investigated using $1x10^5$ autologous B cells infected with vaccinia viruses (v.v. ESO or v.v. WT) as targets. Specific IFN-gamma ELISPOTs produced by CD8 T cells were observed in patient PC-1, but not in patient PC-3, in a range of effector cells as shown in Figure 3B.

Table 3. Correlation between *NY-ESO-1* mRNA expression in primary lesions and NY-ESO-1 antibody response in prostate cancer patients.

mRNA	Ab	No. of Cases
+	+	2
+	-	18
-	+	1[a]
-	-	32
Total		**53**

[a]*NY-ESO-1* mRNA expression was negative in the primary tumor, but was not determined in metastatic lymph nodes (see text).

Table 4. Patient characteristics.

Patient Code	HLA Class I Typing	NY-ESO-1 Status	
		mRNA	Ab
PC1	A*0207,*31;B*46,*56;Cw*01,*04	ND[a]	+
PC2	A*2601;B*4002,*35;Cw*0303,*0304	-[b]	+
PC3	A*0207,*2402;B*46,07*;Cw*01,*07	+	+
PC4	A*0201,*2402;B*4001,*52;Cw*0303,*1202	ND	-

[a]ND, not determined.
[b]*NY-ESO-1* mRNA expression was negative in the primary tumor, but was not determined in metastatic lymph nodes (see text).

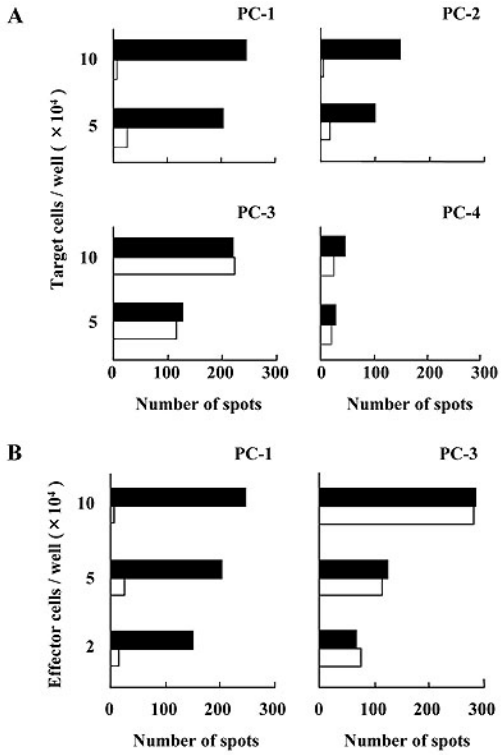

Figure 3. IFN-gamma ELISPOTs produced by CD8 T cells from prostate cancer patients. Purified CD8 T cells were stimulated with autologous CD8-depleted PBLs infected with Ad2/ESO. Responding CD8 T cells were tested for IFN-gamma ELISPOTs in response to autologous B cells infected with vaccinia/*NY-ESO-1* (v.v. ESO) recombinant virus or wild-type vaccinia virus (v.v. WT). (A) Target cell titration. The number of IFN-gamma ELISPOTs produced by $1x10^5$ CD8 T cells in response to $1x10^5$ or $5x10^4$ autologous B cells infected with either v.v. ESO (closed bar) or v.v. WT (open bar). (B) Effector cell titration. The number of IFN-gamma ELISPOTs produced by $1x10^5$, $5x10^4$, $2x10^4$ CD8 T cells against $1x10^5$ autologous B cells infected with either v.v. ESO (closed bar) or v.v. WT (open bar).

Discussion

In the present study, we examined *NY-ESO-1* mRNA expression and immunogenicity in prostate cancer. Tumor specimens used for mRNA preparation were obtained from 53 patients with hard nodular lesions palpable by digital rectal examination. The tumors were clinically classified as stage C (15/53) or D (38/53) prostate cancer. In a panel of 218 patients diagnosed with prostate cancer at the Okayama University Hospital, the frequency of stage A, B, C and D cancers was 4.1%, 12.9%, 18.8% and 64.2% respectively. In a large panel of 1037 patients in Japan, the frequency of stage A, B, C and D prostate cancer at the time of diagnosis was 13.2%, 15.2%, 23.2% and 48.4% respectively (14).

NY-ESO-1 mRNA was highly expressed in advanced prostate cancer (20/53, 38%). No *NY-ESO-1* mRNA expression was observed in 8 BPH specimens. The PCR products were confirmed to be *NY-ESO-1* by nucleotide sequencing, indicating that 35 RT-PCR cycles for the amplification of *NY-ESO-1* mRNA using our sets of primers did not yield false positive signals. *NY-ESO-1* mRNA, but not genomic *NY-ESO-1* DNA or *LAGE-1a* mRNA, which is closely related to *NY-ESO-1* (15), was detected using specific primers and TaqMan probe for

quantitative real-time RT-PCR. A higher frequency of *NY-ESO-1* mRNA expression was observed in prostate cancer with systemic metastasis (stage D2) (15/35, 43%), compared with those with local tumors (stage C) (4/15, 27%) or regional lymph node metastasis (stage D1) (1/3, 33%). Similarly, prostate cancer with higher histological grades (Gleason's score of 8 or more) appeared to have a higher frequency of *NY-ESO-1* mRNA expression. These differences, however, were not statistically significant.

NY-ESO-1 antibody responses were observed in 10 patients with regional lymph node or systemic metastasis out of 218 patients, including 53 whose tumors were examined for *NY-ESO-1* mRNA expression. Seropositivity to NY-ESO-1 was restricted to patients with stage D prostate cancer. NY-ESO-1 antibodies were not detected in the sera from 32 healthy volunteers (15 males and 17 females), substantiating our previous report showing that seropositivity to NY-ESO-1 was restricted to cancer patients (8). Although it was estimated that half of the patients with advanced malignant melanoma expressing *NY-ESO-1* mRNA produced NY-ESO-1 antibodies (8), 2 of 20 patients with *NY-ESO-1* mRNA-positive prostate cancer had antibodies against NY-ESO-1. This low frequency of NY-ESO-1 antibody production was also observed in patients with transitional cell carcinoma (11). Both antibody and CD8 T cell responses to NY-ESO-1 were observed in one of 33 patients (designated PC-2 in Figure 3) whose primary tumor was *NY-ESO-1* mRNA negative, suggesting that a metastatic lesion but not the primary lesion in patient PC-2 expressed NY-ESO-1. No seropositivity was observed in the sera from the other 32 patients with *NY-ESO-1* mRNA-negative primary lesions.

As shown in previous studies with other tumor types (9, 13, 16), strong CD8 T cell responses to NY-ESO-1 were detected in 2 prostate cancer patients with NY-ESO-1 antibodies using recombinant viral constructs encoding *NY-ESO-1* cDNA. In one patient designated PC-1, CD8 T cell responses as well as NY-ESO-1 antibodies were detected over a one-year period. Because the number of known CD8 T cell epitopes identified in the NY-ESO-1 molecule is limited to those binding to HLA-A*0201 and A31 (16, 17) and the CD8 T cell response to NY-ESO-1 is polyclonal (13), the use of recombinant virus is helpful in detecting CD8 T cell responses and identifying new NY-ESO-1 epitopes in patients with NY-ESO-1 antibodies and HLA class I alleles other than HLA-A*0201 and A31 (13). Moreover, this viral system allows the monitoring of CD8 T cell responses in patients of any HLA type vaccinated with various NY-ESO-1 constructs. Vaccination with HLA-A2-restricted peptides was demonstrated to successfully induce a CD8 T cell response to NY-ESO-1 in immunized patients (18). However, less than 10% of the Japanese population carries HLA-A*0201. Based on the data presented in this study, we are now planning a clinical trial with vaccination using NY-ESO-1 protein in advanced prostate cancer, especially in patients with metastatic hormone-refractory prostate cancer in which the effectiveness of current therapies is limited.

Abbreviations

BPH, benign prostatic hyperplasia

Acknowledgements

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References

1. Howe HL, Wingo PA, Thun MJ, Ries LA, Rosenberg HM, Feigal EG, Edwards BK. Annual report to the nation on the status of cancer (1973 through 1998), featuring cancers with recent increasing trends. *J Natl Cancer Inst* 2001; **93**: 824-42. (PMID: 11390532)

2. Kakizoe T, Yamaguchi N, Mitsuhashi F, Koshiji M, Oshima A, Ohtaka M. Cancer statistics in Japan-2001. Tokyo: Foundation for promotion of cancer research; 2001. URL: http://www.ncc.go.jp/en/statistics/2001/index.html

3. Tannock IF, Osoba D, Stockler MR, Ernst DS, Neville AJ, Moore MJ, Armitage GR, Wilson JJ, Venner PM, Coppin CM, Murphy KC. Chemotherapy with mitoxantrone plus prednisone or prednisone alone for symptomatic hormone-resistant prostate cancer: a Canadian randomized trial with palliative endpoints. *J Clin Oncol* 1996; **14**: 1756-64. (PMID: 8656243)

4. Smith DC. Chemotherapy for hormone refractory prostate cancer. *Urol Clin North Am* 1999; **26**: 323-31. (PMID: 10361555)

5. Boon T, Old LJ. Cancer tumor antigens. *Curr Opin Immunol* 1997; **9**: 681-3. (PMID: 9438857)

6. Old LJ, Chen Y-T. New paths in human cancer serology. *J Exp Med* 1998; **187**: 1163-7. (PMID: 9547328)

7. Chen Y-T, Scanlan MJ, Obata Y, Old LJ. Identification of human tumor antigens by serological expression cloning (SEREX). In: Rosenberg SA, editor. Principles and practice of the biologic therapy of cancer. 3rd ed. Philadelphia (PA): Lippincott Williams & Wilkins; 2000. p. 557-70.

8. Stockert E, Jäger E, Chen Y-T, Scanlan MJ, Gout I, Karbach J, Arand M, Knuth A, Old LJ. A survey of the humoral immune response of cancer patients to a panel of human tumor antigens. *J Exp Med* 1998; **187**: 1349-54. (PMID: 9547346)

9. Jäger E, Nagata Y, Gnjatic S, Wada H, Stockert E, Karbach J, Dunbar PR, Lee SY, Jungbluth A, Jäger D, Arand M, Ritter G, Cerundolo V, Dupont B, Chen Y-T, Old LJ, Knuth A. Monitoring CD8 T cell responses to NY-ESO-1: correlation of humoral and cellular immune responses. *Proc Natl Acad Sci USA* 2000; **97**: 4760-5. (PMID: 10781081)

10. Chen Y-T, Scanlan MJ, Sahin U, Türeci Ö, Güre AO, Tsang S, Williamson B, Stockert E, Pfreundschuh M, Old LJ. A testicular antigen aberrantly expressed in human cancers detected by autologous antibody screening. *Proc Natl Acad Sci USA* 1997; **94**: 1914-8. (PMID: 9050879)

11. Kurashige T, Noguchi Y, Saika T, Ono T, Nagata Y, Jungbluth A, Ritter G, Chen Y-T, Stockert E, Tsushima T, Kumon H, Old LJ, Nakayama E. NY-ESO-1 expression and immunogenicity associated with transitional cell carcinoma: correlation with tumor grade. *Cancer Res* 2001; **61**: 4671-4. (PMID: 11406534)

12. Jungbluth A, Antonescu CR, Busam KJ, Iversen K, Kolb D, Coplan K, Chen Y-T, Stockert E, Ladanyi M, Old LJ. Monophasic and biphasic synovial sarcomas abundantly express cancer/testis antigen NY-ESO-1 but not MAGE-A1 or CT7. *Int J Cancer* 2001; **94**: 252-6. (PMID: 11668506)

13. Gnjatic S, Nagata Y, Jäger E, Stockert E, Shankara S, Roberts BL, Mazzara GP, Lee SY, Dunbar PR, Dupont B, Cerundolo V, Ritter G, Chen Y-T, Knuth A, Old LJ. Strategy for monitoring T cell responses to NY-ESO-1 in patients with any HLA class I allele. *Proc Natl Acad Sci USA* 2000; **97**: 10917-22. (PMID: 11005863)

14. Harada M, Nemoto R, Uchida K, Akaza H, Koiso K, Mostofi FK. Histopathological comparison of prostatic adenocarcinoma in Japan and the United States. *Nippon Hinyokika Gakkai Zasshi* 1993; **84**: 1110-8. (PMID: 8345729)

15. Lethe B, Lucas S, Michaux L, De Smet C, Godelaine D, Serrano A, De Plaen E, Boon T. LAGE-1, a new gene with tumor specificity. *Int J Cancer* 1998; **76**: 903-8. (PMID: 9626360)

16. Jäger E, Chen Y-T, Drijfhout JW, Karbach J, Ringhoffer M, Jäger D, Arand M, Wada H, Noguchi Y, Stockert E, Old LJ, Knuth A. Simultaneous humoral and cellular immune response against cancer-testis antigen NY-ESO-1: definition of human histocompatibility leukocyte antigen (HLA)-A2-binding peptide epitopes. *J Exp Med* 1998; **187**: 265-70. (PMID: 9432985)

17. Wang RF, Johnston SL, Zeng G, Topalian SL, Schwartzentruber DJ, Rosenberg SA. A breast and melanoma-shared tumor antigen: T cell responses to antigenic peptides translated from different open reading frames. *J Immunol* 1998; **161**: 3598-606. (PMID: 9759882)

18. Jäger E, Gnjatic S, Nagata Y, Stockert E, Jäger D, Karbach J, Neumann A, Rieckenberg J, Chen Y-T, Ritter G, Hoffman E, Arand M, Old LJ, Knuth A. Induction of primary NY-ESO-1 immunity: CD8+ T lymphocyte and antibody responses in peptide-vaccinated patients with NY-ESO-1+ cancer. *Proc Natl Acad Sci USA* 2000; **97**: 12198-203. (PMID: 11027314)

19. Jewett HJ. The present status of radical prostatectomy for stages A and B prostatic cancer. *Urol Clin North Am* 1975; **2**: 105-24. (PMID: 1128746)

20. Gleason DF. Classification of prostatic carcinomas. *Cancer Chemother Rep* 1966; **50**: 125-8. (PMID: 5948714)

21. Sauza DW, Armentano D. Novel cloning method for recombinant adenovirus construction in Escherichia coli. *BioTechniques* 1999; **26**: 502-8. (PMID: 10090992)

Materials and methods

Patients, sera and tumor specimens

Patients were 218 individuals who visited the Department of Urology, Okayama University Hospital, from January 1999 to December 2002. Patients underwent transurethral resection of the prostate or transrectal needle biopsy and were diagnosed histologically with prostate cancer. The mean age at diagnosis was 72 (range 53 to 94). All patients had tumors at the time of serum collection. Clinical staging of prostate cancer was assessed according to the consensus report (19). The Gleason's score was determined according to the standard criteria (20).

Sufficient amount of tumor specimens for analysis of *NY-ESO-1* mRNA expression was obtained from 53 patients with hard nodular lesions, palpable on digital rectal examination, which were recognized as low echoic areas on transrectal ultrasonography. The mean age at diagnosis in these 53 patients was 74 (range 57 to 94). Serum prostate specific antigen levels for these 53 patients ranged from 7 to 5974 ng/ml with a median value of 208 ng/ml. A bone metastatic lesion was obtained from a patient who underwent surgery due to sudden paraplegia. Specimens of benign prostatic hyperplasia were obtained from 8 patients by transurethral resection. Informed consent was obtained from all the patients for the use of specimens and sera.

RT-PCR analysis

mRNA was isolated from frozen tumor specimens using the QuickPrep Micro mRNA Purification Kit (Pharmacia, Uppsala, Sweden). Isolated mRNA was subjected to cDNA synthesis using the First-Strand cDNA Synthesis Kit (Pharmacia). Primers for RT-PCR were: ESO1-1, 5'-AGTTCTACCTCGCCATGCCT-3' and ESO1-2, 5'-TCCTCCTCCAGCGACAAACAA-3'. The amplification program for *NY-ESO-1* was 1 min at 94°C, 1 min at 62°C and 1.5 min at 72°C for 35 cycles, after initial denaturation at 94°C for 1 min. These cycles were followed by a 10 min elongation step at 72°C. The PCR products (385 bp) were analyzed on an 0.8% agarose gel.

Quantitative real-time RT-PCR

Real-time RT-PCR was run on the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Foster city, CA, USA) to amplify the *NY-ESO-1* gene product. The forward primer was 5'-GCTGAATGGATGCTGCAGA-3' from exon 1 and the reverse primer was 5'-CTGGAGACAGGAGCTGATGGA-3' from exon 3, leading to an amplicon of 250 bp in length. The TaqMan probe (5'-FAM-TGTGTCCGGCAACATACTGACTATCCGA-TAMRA-3') was designed to span the junction of exon 2 and 3. The specificity of the primers and TaqMan probe was confirmed using human genomic DNA (BD Biosciences Clontech, Palo Alto, CA, USA) and *LAGE-1a* cDNA cloned from human testis cDNA. Glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) was amplified as an endogenous control using TaqMan human GAPDH control reagents (Applied Biosystems). Human testis cDNA was purchased from BD Biosciences Clontech. The threshold cycle (Ct) value was determined as the cycle at which the fluorescence of the reaction first arose above the background. The Ct value was regarded as infinite when no fluorescence rose from the background even after 40 amplification cycles. The Ct value obtained for the endogenous control was subtracted from the Ct value for *NY-ESO-1* for each specimen (DELTACt = Ct of *NY-ESO-1* - Ct of *GAPDH*). The expression level of *NY-ESO-1* mRNA in specimens relative to that in normal testis was calculated by subtracting the DELTACt value for normal testis from the DELTACt value obtained for

specimens (DELTADELTACt = DELTACt of specimens - DELTACt of the normal testis), and the relative expression level was calculated as $2^{-DELTADELTACt}$.

ELISA

ELISAs were performed using recombinant NY-ESO-1 protein as the antigen as described (8).

Generation of viral vectors

The adenoviral construct encoding *NY-ESO-1* (Ad2/ESO) was provided by Genzyme Corporation (Cambridge, MA, USA). The vaccinia virus construct encoding *NY-ESO-1* (v.v. ESO) was provided by Therion Biologics (Cambridge, MA, USA). Details have been described elsewhere (13, 21).

Infection of antigen presenting cells (APCs) or target cells with recombinant viruses

For APCs, CD8 T cell-depleted peripheral blood lymphocytes (PBLs) were infected with adenovirus constructs at 100 IU/cell for 20 hr at 37°C in 300 µl X-VIVO-15 (Bio-Whittaker, Walkersville, MA, USA). For target cells, $1x10^6$ PBLs cultured with 200 U/ml human rIL-6 (Peprotech, London, England) and 10 ng/ml human rIL-12 (Peprotech) for 1 week were infected with vaccinia/*NY-ESO-1* recombinant virus (v.v. ESO) or wild-type vaccinia virus (v.v. WT) at 30 pfu/cell for 20 hr at 37°C in 300 µl X-VIVO-15.

***In vitro* sensitization of CD8 T cells with adenoviral constructs**

CD8 T cells were purified from PBLs using antibody-coated magnetic beads (Miltenyi Biotec, Auburn, CA, USA). $5x10^5$ CD8 T cells were co-cultured with irradiated $2x10^6$ APCs infected with adenovirus constructs in 24-well plates (Becton Dickinson, Franklin Lakes, NJ, USA) in RPMI 1640 with 5% human AB serum (Sigma, St. Louis, MO, USA), 2 mM L-glutamine, 100 U/ml penicillin, 100 µg/ml streptomycin, and 1% nonessential amino acids for 10 days at 37°C in 5% CO_2 atmosphere. 10 U/ml human rIL-2 (Takeda, Osaka, Japan) and 20 ng/ml human rIL-7 (Peprotech) were added every 3 days.

ELISPOT assays

Responding CD8 T cells ($2x10^4$ to $1x10^5$) and target cells ($5x10^4$ or $1x10^5$) were cultured in 96-well nitrocellulose plates (Millipore, Bedford, MA, USA) precoated with 2 µg/ml anti-human IFN-gamma mAb (1-DIK; Mabtech, Stockholm, Sweden) for 20 hr at 37°C in RPMI 1640 without IL-2 and human serum. After washing, rabbit anti-human IFN-gamma serum diluted 1:800 with phosphate buffered saline was added and incubated for 2 hr at 37°C. After washing extensively, goat anti-rabbit IgG serum conjugated with alkaline phosphatase (Southern Biotechnology, Birmingham, AL, USA) diluted 1:2000 was added and the plates were incubated for 1 hr at 37°C. After washing, substrate (AP conjugate substrate kit; Bio-Rad Laboratories, Hercules, CA, USA) was added and the plates were incubated for 15 min. Spots were counted under a microscope after washing the plates.

Contact

Address correspondence to:

Yuji Noguchi
Department of Immunology
Okayama University Graduate School of Medicine and Dentistry
2-5-1 Shikata-cho Okayama 700-8558
Japan
Tel.: + 81 86 235-7188
Fax: + 81 86 235-7193
E-mail: noguchi@md.okayama-u.ac.jp